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A RNASE FROM YEAST ASSOCIATED WITH THE 40S RIBOSOMAL SUBUNIT.
AU SCHULZ-HARDER B; KAEUFER N; SWIDA U
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ribonuclease enzymes present in yeast

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A RIBONUCLEASE FROM YEAST ASSOCIATED WITH THE 40 S RIBOSOMAL SUBUNIT

BERND SCHULZ-HARDER *, NORBERT KÄUFER and ULRIKE SWIDA

Freie Universität Berlin, Fachbereich Biologie WE 3, Institut für Biochemie und Molekularbiologie, Ehrenbergstr. 26-28, D-1000 Berlin 33 (Germany)

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Key words: Ribosomal ribonuclease; Ribosomal subunit; (S. cerevisiae, Wheat germ)

Summary

1. Autodegradation of yeast ribosomes is due to a 'latent' ribonuclease which is associated with the 40 S ribosomal subunit.

2. The ribonuclease was extracted in the presence of EDTA from ribosomes and purified 118-fold by protamine sulphate precipitation, $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose.

3. The optimum pH for this enzyme is 5 to 6.5, while the optimum temperature is 45 to 50°C. Incubation for 10 min at 60°C caused a reduction in enzyme activity of 70%.

4. The ribonuclease has an endonucleolytic activity against rRNA, tRNA, poly(A), poly(U) and poly(C) but does not degrade poly(G) or DNA. It hydrolyzes the homopolymers to nucleoside 3'-phosphates.

5. Zn^{2+}, Mn^{2+}, heparin, glutathione and *p*-chloromercuribenzoate inhibit the ribonuclease, while Na^+, K^+, EDTA and spermidine have only little or no effect.

6. It binds tightly to yeast ribosomes but only loosely to ribonuclease-free wheat germ ribosomes.

7. Polyribosomes possess less autodegradation activity than monoribosomes, isolated from the same homogenate.

Intr duction

In order to control the RNA content of the cell, microorganisms are provided with a number of ribonucleases [1]. Prokaryotes and eukaryotes possess

* Present address: National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

ribonucleases, ass ciated with rib somes. Since the work of Neu and Heppel [2–5] there has been c nsiderable d ubt expressed concerning the binding of rib nuclease I to rib somes in intact *Escherchia coli* cells. The enzyme has been shown to be periplasmic, in viv , and the ass ciation to the ribosomes occurs during disrupti n of the cell. H wever, other workers [6–8] have suggested that the association of ribonuclease to rat liver ribosomes is no artefact. This discrepancy could be due to the heterogeneity of prokaryotic and eukaryotic ribosomes. Our results also support the assumption that yeast rib nuclease is bound to ribosomes in the intact cell.

Yeast ribonucleases have been extracted and purified from whole cells [9], microsomal fraction [10,11] or the postribosomal supernate [12]. In contrast to the ribonuclease activity found in the cytoplasm, the ribosomal rib nuclease activity increases when the cells enter the stationary growth phase [13]. Recently, an exonuclease has been isolated from ribosomes of *Saccharomyces cerevisiae* which hydrolyzes RNA in the 5'- to 3'-direction [14], and two ribonucleases H which specifically degrade RNA in RNA–DNA hybrids have been highly purified [15,16].

In this paper we report on the isolation and the properties of a rib nuclease from yeast, which is associated with the 40 S ribosomal subunit, and discuss its role in the RNA catabolism.

Materials and Methods

Strains and growth conditions. The diploid strain 211 or the tetraploid strain 2200 of *S. cerevisiae* [17] were used. The cells were grown in a medium containing 0.5% peptone, 1% Difco yeast extract and 2% glucose at 30°C, and harvested in the stationary growth phase.

Preparation of yeast ribosomes. The preparation of polyribosomes was described previously [18]. For the isolation of ribosomes yeast cells were suspended in buffer A (30 mM Tris-HCl, pH 7.5, 200 mM KCl, 5 mM $MgCl_2$, 0.25 mM EDTA, 6 mM 2-mercaptoethanol) containing 20% (v/v) glycerol. The cell suspension (40 ml) was poured into a French Pressure Cell (American Instrument Co.) and frozen in an ethanol/solid CO_2 bath. The frozen mixture was then forced through the outlet (inside diameter 1.4 mm) at a pressure of 20 000 lb/inch^2. Following the disruption of the cells, the homogenate was centrifuged at 18 000 × *g* for 10 min in a Sorvall centrifuge, and the p stmitochondrial supernatant was made 1% in Brij 58 (Serva, Heidelberg, F.R.G.). 20 ml portions of this supernatant were layered on 14 ml sucrose cushions f 1 M sucrose in buffer A. After centrifugation at 131 000 × *g* for 18 h at 2°C (SW 27 rotor), the supernatants were removed by aspiration and the pellets were quickly rinsed with distilled water and then resuspended in buffer B (30 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.25 mM EDTA, 6 mM 2-mercaptoethanol). The absorbance of the ribosomal suspension was adjusted to 300–600 A_{260nm}.

Preparation of wheat germ ribosomes. The 30 000 × *g* supernatant (S-30) of wheat germ was prepared according to the method of Roberts and Paterson [19], or the homogenisation of wheat germ was carried out with buffer A containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$.

Preparation of ribosomal subunits. 200—300 A_{260nm} f yeast ribosomes were layered on a linear sucrose gradient (15—30% w/w) in buffer A c ntaining 500 mM KCl), and centrifuged at 131 000 × *g* f r 8 h (SW 27 rotor, 15°C). The subunits were pooled, pelleted by centrifugation, and resuspended in buffer B. The absorbance f the 60 S subunit suspensi n was adjusted to 300 A_{260nm} and that of the 40 S subunit to 170 A_{260nm}.

Isolation of yeast ribonuclease. About 1400 mg purified yeast ribosomes were incubated in buffer B containing 10 mM EDTA for 45 min at 35°C. After centrifugation at 30 000 × *g* for 10 min, protamine sulphate was added to the supernatant (250 μg/ml), and the mixture was stirred for 30 min at 0°C. The precipitate was removed by centrifugation at 30 000 × *g* for 10 min, and solid $(NH_4)_2SO_4$ was added to the supernatant. The precipitate between 40% and 80% saturation was collected by centrifugation, dissolved in 8 ml buffer C (30 mM Tris-HCl, pH 7.5, 6 mM 2-mercaptoethanol), and dialyzed overnight against 4 l of the same buffer with one change. The $(NH_4)_2SO_4$ fraction was chromatographed on a DEAE-cellulose column (2 × 10 cm; flow rate, 35 ml/h), equilibrated with buffer C. After washing with buffer C (150 ml) the column was eluted with a linear gradient of 0—200 mM KCl in buffer C. The fractions c ntaining the ribonuclease activity were pooled and concentrated with polyethyleneglycol) (type 20 000, Serva).

Preparation of ribosomal RNA. Ribosomal RNA (rRNA) was prepared from yeast rib s mes according to the method of Brawerman [20].

Preparation of spheroplasts. Spheroplasts were prepared by a modified method of Holz et al. [21]. *S. cerevisiae* was grown in a medium containing 2% sucrose, 1% Difco yeast extract and 0.5% peptone under aerobic conditions. The cells were harvested in the stationary phase and incubated for 15 min at 30°C in 100 mM Tris-HCl, pH 8.0 and 20 mM 2-mercaptoethanol. After washing, 10^{11} cells were incubated in 10 ml snail enzyme (Industrie Biologique Francaise, Gennevilliers), 20 ml 50 mM 2-mercaptoethanol and 40 ml 1.2 M KCl, and the formation of spheroplasts was followed under a light microscope. The snail enzyme was eliminated by centrifuging at 700 × *g* for 2 min and washing three times with an osmotic stabilizer (600 mM mannitol, 2% (w/v) glucose, 5 mM sodium citrate, pH 6.5). The pellet was then resuspended in the osm tic stabilizer diluted 1 : 2.5 (10^{10} cells in 15 ml), and lysis was performed by means f a Potter-Elvehjem homogenizer (ten strokes).

Autodegradation assay. Autodegradation of ribosomes or subunits was assayed in a 0.1 ml of a mixture containing 7—11 mg/ml ribosomes and 10 mM EDTA in buffer B. The incubation was performed at 35°C. In order to determine the amount of acid-soluble material, 18-μl samples were removed from the incubation mixture at various times and mixed with 80 μl of 25% perchloric acid and 0.75% uranyl acetate. After dilution to 1 ml with distilled water and centrifugation at 5400 × *g* for 5 min (Bio-Dynamics Select-a-Fuge 24) the absorbances of the supernatants were measured against a blank at 260 nm.

Ribonuclease assay. The ribonuclease activity was assayed in a 0.1 ml of a mixture containing 2.5 to 5 mg/ml RNA in buffer C. The acid-soluble material was detected as described above.

Results

Isolation of ribonuclease from ribosomes

Yeast cells harvested at the stationary growth phase were homogenized with a French Press, this having been shown to be a gentle method for the isolation of cell organelles from yeast, preserving the structure of polyribosomes [18] and nuclei [22–24]. In order to minimize artificial binding of ribonucleases to ribosomes, we used a buffer with appropriate ionic strength for the homogenisation.

The purification processes are summarized in Table I. Following the extraction of the ribonuclease from the ribosomes by EDTA, the ribonucleic acids were precipitated with protamine sulphate, and the ribonuclease was found to be precipitable with 40–80% (saturation) ammonium sulphate. After the DEAE chromatography (Fig. 1) the yield of ribonuclease was 3% of the purified ribosomes, and the specific activity increased 118-fold.

Properties of ribonuclease

Unlike other yeast ribonucleases [9,10] the pH optimum for this ribosomal enzyme was found to be in the range of pH 5 to 6.5, the temperature optimum being 45 to 50°C. However, to study the physiological role of the ribonuclease in the cell, we had chosen pH 7.5 and a temperature of 35°C in our investigations. In order to investigate the heat-stability of the ribonuclease, the enzyme was maintained at various temperatures for 10 min, chilled rapidly to 0°C, and assayed for ribonuclease activity. At 60°C the enzyme lost 70% of activity.

The effects of various agents on the ribonuclease activity are indicated in Table II. Glutathione, heparin and *p*-chloromercuribenzoate inhibited the reaction, while EDTA and spermidine had only little effect. Nevertheless, spermidine affected the autodegradation of ribosomes more than the activity of the isolated enzyme, which is probably due to the stabilization of the ribosome structure by the polyamine (result not shown).

While 5 mM $MgCl_2$ prevented the autodegradation of ribosomes, it inhibited the ribonuclease activity to an extent of only 18%. A stronger inhibition was achieved by $MnCl_2$ (0.5 mM) and $ZnSO_4$ (0.5 mM), which reduced the activity to 32% or blocked the enzyme completely. In contrast to the divalent cations, KCl and NaCl did not influence the reaction.

Our investigation of the substrate specifity revealed a high affinity of the

TABLE I

SUMMARY OF PURIFICATION OF YEAST RIBONUCLEASE

Activity: 1 unit = 0.1 A_{260nm}/min per ml.

Fraction	Total protein (mg)	Total activity (unit)	Spec. act. (unit/mg protein)	Yield (%)
Suspension of ribosomes	690	13 860	20	100
EDTA-extraction and protamine sulphate precipitation	80	5 320	67	38
Ammonium sulphate fractionation	8	2 950	369	21
DEAE-cellulose chromatography	0.17	400	2354	3

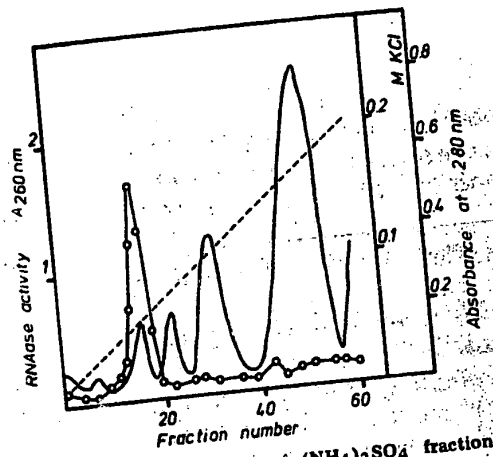

Fig. 1. Chromatography of $(NH_4)_2SO_4$ fraction on DEAE-cellulose as described in Materials and Methods. 1 ml fractions were collected and tested for ribonuclease activity. o———o, ribonuclease activity; ———, A_{260nm}.

ribonuclease to rRNA. The enzyme completely degraded rRNA after a short incubation time, while it slowly hydrolyzed tRNA and had no effect on DNA. Among the synthetic homopolynucleotides, poly(A) and poly(U) were degraded more quickly than poly(C), while poly(G) was resistant to the attack of the enzyme. An exonuclease (RNAase CL) with similar substrate specifities was extracted and purified from *Candida lipolytica* by Imada et al. [25].

Since ribosomal yeast ribonuclease caused a slow but significant breakdown of polyribosomes (Fig. 4) we expected an endonucleolytical cleavage of RNA, and this was confirmed by sedimentation analysis of the partial digests of rRNA (Fig. 2). Ribosomal RNA was incubated with yeast ribonuclease for various times, and sedimented on sucrose gradients. After 10 min incubation

TABLE II

EFFECT OF AGENTS ON RIBONUCLEASE ACTIVITY

The relative activity was assayed using Tris-HCl buffer, pH 7.5, at a final concentration of 30 mM.

Agents	Concentration (mM)	Relative activity (%)
None (control)		100
EDTA	5	96
p-Chloromercuribenzoate	1	65
	5	20
Glutathione	1	100
	2	74
	3	0
Spermidine	0.5	98
	10	87
Heparin	0.005	96
	0.05	12
	0.5	12

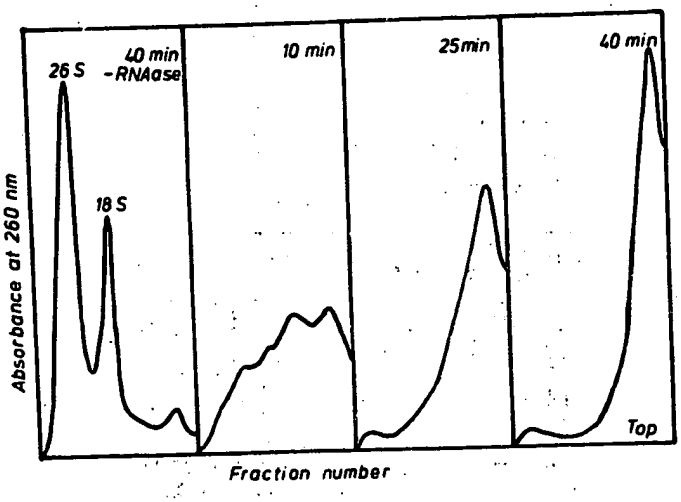

Fig. 2. Sedimentation analysis of rRNA after incubation with yeast ribonuclease for 10 min, 25 min, 40 min and without ribonuclease at 35°C. Centrifugation was performed in a SW 27 rotor with 10—30% (w/v) sucrose gradients containing 20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA and 0.5% SDS, for 15 h at 131 000 × *g* (22°C).

the pattern shows a heterogeneous size distribution of cleaved rRNA, it is shifted to smaller sizes after 25 to 40 min. The amount of mono- and oligonucleotides, which accumulate on top of the gradient, increased slowly. To determine the mononucleotides, homopolynucleotides were incubated with ribonuclease for 22 h. The degradation products analyzed by thin-layer chromatography [26] showed only nucleoside 3′-phosphates.

Localization of ribonuclease

The distribution of ribonuclease activity on ribosomal subunits is demonstrated in Fig. 3. The association of the ribonuclease with the 40 S subunit and

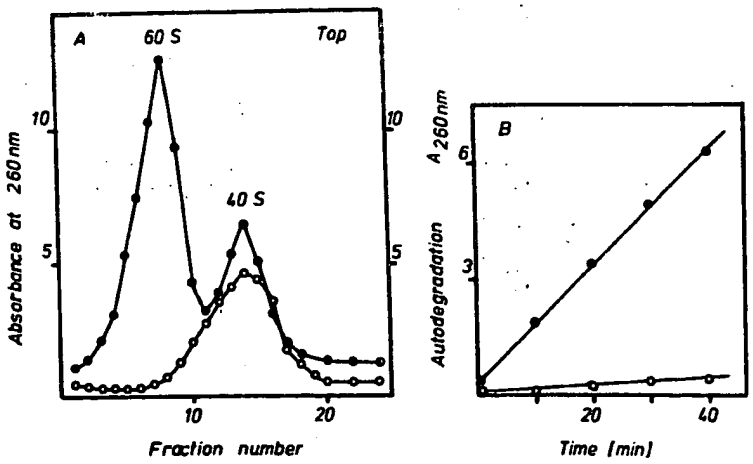

Fig. 3. (A) Centrifugation of ribosomes in a linear 10—30% (w/v) sucrose gradient containing 30 mM Tris-HCl, pH 7.5, 500 mM KCl, 5 mM $MgCl_2$ and 6 mM 2-mercaptoethanol. (5 h at 131 000 × *g* in a SW 27 rotor (2°C). The 1 ml fractions were tested by autodegradation in 10 mM EDTA, and the acid-soluble material was measured at 260 nm after 30 min incubation at 35°C. ●——●, ribosomal subunits; ○——○, autodegradation. (B) Autodegradation activity of subunits from yeast ribosomes. ●——●, 40 S subunits; ○——○, 60 S subunits. The autodegradation assay was performed with 10 mM EDTA at 35°C for various lengths of time.

its activati n by EDTA resembles the behaviour of rib nuclease I of *E. coli* 30 S ribosomal subunit [27,28]. But Neu and Heppel [2—5] could show that an enzyme with similar ribonuclease activity was released from spheroplasts after EDTA treatment, which allowed them to infer ribonuclease I to be localized in the periplasmic space. In order to elucidate whether yeast ribonuclease is also localized between the cell membrane and the cell wall, we treated spheroplasts with 2 mM EDTA, and the EDTA wash, the lysate and the postmitochondrial supernatant were assayed for ribonuclease activity. The lysate (100%) and the postmitochondrial supernatant (74%) showed a considerably higher activity than did the EDTA wash (23%). (Similar activities were obtained when the spheroplasts were washed without EDTA.) The activity in the EDTA wash is probably due to spheroplasts, which were burst during incubation and centrifugation, or could be due to an EDTA-insensitive ribonuclease which is found in the snail enzyme (result not shown). To avoid contamination with this ribonuclease, the spheroplasts had to be washed with buffer before treatment with EDTA. Although, the buffer did not contain EDTA or high salt concentration, one cannot absolutely exclude that periplasmic enzymes were eliminated.

A further possibility for artificial association could be the contamination of ribosomes with a ribonuclease, enclosed in intracellular vesicles, in vivo, and liberated during disruption of cells. However, a ribonuclease with binding affinity to ribosomes, localized in intra- or extracellular spaces, should also be detectable in other subcellular fractions. Indeed, we were able to find a ribonuclease activity in the 48 000 × *g* pellet of the yeast homogenate, which could originate from the periplasmic space or intracellular membrane vesicles. But this ribonuclease activity is inhibited by the postribosomal supernatant, while ribosomal ribonuclease is not (result not shown).

Binding of ribonuclease to ribosomes

Since it was difficult to prove the lack of yeast ribonuclease in the periplasmic space, we focussed our attention on the specific binding of the ribo-

TABLE III

BINDING OF RIBONUCLEASES TO RIBOSOMES

Yeast ribosomes (derived from logarithmic growing cells) were mixed with yeast ribonuclease or ribonuclease A. After centrifugation through a sucrose cushion, the relative autodegradation activity of the ribosomes was assayed.

	1st centrifugation (% activity)	2nd centrifugation (% activity)
Ribosomes — RNAase	78	73
Ribosomes + yeast RNAase	100	100
Ribosomes + RNAase A (0.01 μg/ml)	81	71
Ribosomes + RNAase A (0.05 μg/ml)	95	72
Control:		
Ribosomes — RNAase not washed	78	72
Ribosomes with addition of RNAase A (0.01 μg/ml) to the autodegradation assay	156	—

nuclease to yeast ribosomes. The binding of rib nuclease to rib somes was investigated with rib somes derived from logarithmic growing cells, which have little ribonuclease activity, and was c mpared with the binding of ribonuclease A. As is demonstrated in Table III, yeast ribonuclease binds very tightly to yeast rib s mes, while rib nuclease A was removed by washing with buffer A made 1% in Brij 58.

Furthermore, unspecifically bound ribonuclease should bind to heterol g us ribosomes just as well as to homologous ribosomes. Concerning this aspect, it was an important finding that ribosomes derived from wheat germ and *Dictyostelium discoideum* possess no autodegradation activity (result not shown). If the S-30 of wheat germ, prepared as described by Roberts and Paterson [19], was mixed with yeast ribonuclease and centrifuged, the pelleted ribosomes showed a high autodegradation activity. But ribosomes mixed with ribonuclease in buffer A made 1% in Brij 58 and centrifuged through a 1 M sucrose cushion lost 92% of the ribonuclease activity. This result indicates a lo se ass - ciation between yeast ribonuclease and wheat germ ribosomes, and makes it likely that the tight interaction with homologous ribosomes is very specific.

The affinity of yeast ribonuclease to polyribosomes and monoribosomes

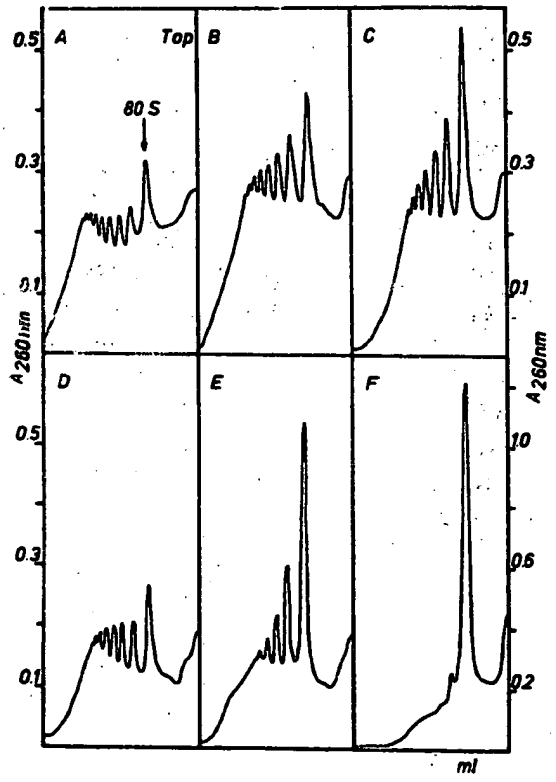

Fig. 4. Sedimentation analysis of polyribosomes after incubation with 1 mM cycloheximide for 1 min, 15 min and 30 min at 35°C (A—C). Polyribosomes incubated with yeast ribonuclease for 1 min and 15 min at 35°C (D,E). Polyribosomes incubated with ribonuclease A (0.05 μg/ml) for 15 min at 35°C (F). (0.05 μg/ml ribonuclease A had the same activity as yeast ribonuclease detected by the ribonuclease assay.) Centrifugation was performed in a SW 27 rotor with 20—50% (w/v) sucrose gradients, containing 30 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$ and 6 mM 2-mercaptoethan l, for 5 h at 131 000 × g (2°C).

seems to be different. W found that polyrib somes p ssess less autodegradati n activity than monoribosomes, isolated from the same h mogenate (specific activity: polyrib s mes = 1.25 A_{260nm}/min per mg, monoribosomes = 1.48 A_{260nm}/min per mg). A similar finding has been reported previously by Bransgrove and C squer [8]. H wever, it was impossible to isolate polyribosomes with ut any ribonuclease activity. In Fig. 4 A—C it is shown that the negligible increase of monoribosomes during the incubation of polyribosomes in vitro, is due to a low endogenous ribonuclease activity, and not to the running-off of rib somes, which was prevented by cycloheximide. When polyribosomes were mixed with additional yeast ribonuclease, the degradation was raised only a little (Fig. 4 D, E), while an equal amount of ribonuclease A led to a complete breakdown of polyribosomes within 15 min of incubation (Fig. 4 F).

Discussion

The autodegradation of yeast ribosomes is due to a 'latent' endoribonuclease, which is associated with the 40 S ribosomal subunit. The rinonuclease hydrolyzes rRNA, tRNA, poly(A), poly(U) and poly(C) to nucleoside 3'-phosphates but does not degrade poly(G) or DNA. It requires no metal ions for its activity and it is not affected by EDTA. Zn^{2+} and Mn^{2+} as well as heparin, glutathione and *p*-chloromercuribenzoate inhibit the reaction.

In addition to yeast [10,11,13], the presence of ribosomal ribonucleases has been described in other cells e.g. rat liver [6—8], rat spleen [29], *Nicotina tabacum* [30] and *E. coli* [27,28]. On the other hand ribosomes isolated from wheat germ and *Dictyostelium discoideum* possess no autodegradation activity. The fact that eukaryotic ribosomes already show very different affinities to ribonucleases makes it difficult to compare eukaryotic (yeast) and prokaryotic (*E. coli*) ribosomes. However, it is obvious that the binding of yeast ribonuclease to the 40 S ribosomal subunit and its activation by EDTA resembles the behaviour of ribonuclease I, which was found to be periplasmic [2—5]. But since only half of the ribosomal ribonuclease was released from spheroplasts (stationary *E. coli* cells) and the remaining ribosomal ribonuclease is distinguishable from the periplasmic enzyme by its different affinity to DEAE [2], existence of two ribosomal ribonucleases should be considered. Indeed, there is also evidence for the occurrence of different ribosomal ribonucleases in yeast cells derived from the logarithmic or stationary growth phase [31].

It is well known, that the rise in the ribonuclease activity during the growth of yeast is accompanied by a decrease of the RNA content [11,32]. Therefore it seems likely that this ribonuclease is involved in the RNA catabolism, and that the specific interaction between yeast ribonuclease and yeast ribosomes, causing an inactivation of the enzyme, could be part of a regulatory mechanism for ribosome degradation.

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L15 61 S L1 (P) L2 (P) L7
L16 8 S L15 (P) L12
L17 6 DUP REM L16 (2 DUPLICATES REMOVED)
L18 741 S L1 (25A) L2
L19 623 S L1 (15A) L2
L20 299598 S CANDIDA OR TORULA OR SACCHAROMYCES OR BREWER?
L21 741 S L1 (25A) L2
L22 95414 S L1 (25A) L20
L23 328 S L2 (25A) L20
L24 13117 S AUTOLYS? OR AUTOLYS?
L25 0 S L23 (25A) L24